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HTLV-I specific IFN-γ + CD8 + lymphocytes correlate with the proviral load in peripheral blood of infected individuals

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Abstract

Human T lymphotropic virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is an inflammatory neurological disease caused by HTLV-I infection. It has been shown that HAM/TSP patients have high proviral loads and an extraordinarily high frequency of circulating CD8 + cytotoxic T lymphocytes specific for HTLV-I in their peripheral blood when compared to asymptomatic HTLV-I carriers (AC). We have previously described an intracellular cytokine detection assay, in which interferon-γ (IFN-γ) + CD8 + lymphocytes are specific for HTLV-I in infected individuals. Here, we have established a competitive polymerase chain reaction assay to measure the proviral load of patients and investigate a potential relationship between proviral load and virus-specific CD8 + lymphocytes. Genomic DNA was extracted from peripheral blood lymphocytes (PBL) from eight HAM/TSP patients and seven AC for the measurement of HTLV-I measuring proviral loads. The same PBL were analyzed for intracellular IFN-γ expression by flow cytometry. In the HAM/TSP patients and AC, the average proviral loads were 34,482 and 9784 copy/μg DNA ($P = 0.021$), and the average of IFN-γ + CD8 + lymphocytes in total PBL were 1.47 and 0.08% ($P = 0.001$), respectively. It was confirmed that HAM/TSP patients have both high proviral loads and increased HTLV-I-specific CD8 + lymphocytes. Furthermore, we found a positive correlation between both factors in the patients with HAM/TSP ($P = 0.044$) but not in the AC ($P = 0.508$). These findings suggest that the high number of HTLV-I-specific lymphocytes may result from the increased proviral load in HAM/TSP patients.

Keywords: Human T lymphotropic virus type I (HTLV-I); HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP); Proviral load; CD8 + lymphocytes

1. Introduction

Human T lymphotropic virus type I (HTLV-I) is a human retrovirus well known as the causative agent for adult T-cell leukemia/lymphoma (ATL) (Uchiyama et al., 1997) and a slowly progressive neurological disorder, termed HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al., 1985; Osame et al., 1986). HAM/TSP patients have upper motor neuron signs and mild sensory and sphincter dysfunction (Osame et al., 1987). Pathologically, HAM/TSP is characterized by perivascular infiltration of inflammatory cells with demyelination; lesions are most prominent in the thoracic spinal cord (Akizuki et al., 1987). Certain immunological parameters have been shown to be abnormal in HAM/TSP patients in comparison with HTLV-I-infected asymptomatic carriers (AC) and healthy controls (HC). These include high HTLV-I proviral loads in peripheral blood lymphocytes (PBL) (Yoshida et al., 1989; Kubota et al., 1993; Nagai et al., 1998), high antibody titers to HTLV-I in both sera and cerebrospinal fluid (CSF) (Osame et al., 1987) and increased spontaneous lymphoproliferation in vitro (Itoyama et al., 1988; Jacobson et al., 1988; Usuku et al., 1988). In addition, HAM/TSP patients show extraordinarily high levels of circulating HTLV-I-specific CD8 + cytotoxic T lymphocytes (CTL), which are specific for the

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HTLV-I Tax 11–19 peptide in human leukocyte antigen (HLA)-A2 patients (Jacobson et al., 1990; Kannagi et al., 1991; Koenig et al., 1993). The frequency of HTLV-I Tax-specific CD8 + CTL is as high as 1 in 75 to 1 in 320 CD8 + cells in PBL of HAM/TSP patients (Elovaara et al., 1993). The frequency of these lymphocytes in CSF cells from a patient with HAM/TSP is similar in magnitude to those in PBL (Jacobson et al., 1992). Immunohistochemical analysis of the spinal cord lesions reveals the expression of MHC class I molecules and an accumulation of infiltrating CD4 + and CD8 + lymphocytes. These CD8 + lymphocytes predominate with duration of illness (Moore et al., 1989; Umehara et al., 1993). Molecular biological studies have shown the presence of the HTLV-I genome and its expression in the affected lesions of HAM/TSP patients (Kira et al., 1992; Hara et al., 1994; Kubota et al., 1994; Lehky et al., 1995; Moritoyo et al., 1996). Therefore, we have hypothesized that the increased number of the virus-specific CD8 + cells might play a critical role in the inflammatory responses in HAM/TSP patients (Jacobson, 1996).

We have previously reported that HAM/TSP patients have increased numbers of IFN-γ + CD8 + cells in the PBL than AC by a flow cytometric assay combined with intracellular cytokine staining (Kubota et al., 1998). The IFN-γ production from CD8 + cells was blocked by an addition of anti-major histocompatibility complex (MHC) class I antibody and was observed when antigen-presenting cells prepulsed with HTLV-I peptide or autologous CD4 + cells were added. Since CD4 + cells are the main reservoir for HTLV-I in vivo (Richardson et al., 1990), these data suggest that these CD8 + cells produce the cytokine through recognition of HTLV-I antigens bound to MHC class I molecules on the infected CD4 + cells. Thus, we can detect HTLV-I-specific IFN-γ + CD8 + cells in the PBL of the infected individuals using this assay.

In patients with human immunodeficiency virus type 1 (HIV-1), another chronic retroviral infection, it has been demonstrated that the frequency of HIV-1-specific lymphocytes inversely correlates with the viral burden (Greenough et al., 1997; Ogg et al., 1998). However, in HTLV-I infection, it is still uncertain if the virus-specific CD8 + lymphocytes have any relationship to the proviral load in HTLV-I-infected individuals. To address this issue, we employed a competitive polymerase chain reaction (PCR) technique for measuring the proviral load and a flow cytometric analysis combined with intracellular IFN-γ staining for detecting HTLV-I-specific CD8 + lymphocytes in the same series of samples from HTLV-I-infected individuals, which included eight HAM/TSP patients and seven AC. We have found that HAM/TSP patients, when compared to AC, have both high proviral loads and increased HTLV-I-specific CD8 + lymphocytes in the PBL. Moreover, we showed that the virus-specific CD8 + lymphocytes positively correlated with the proviral loads in PBL of the HAM/TSP patients. These data suggests that the high number of HTLV-I-specific lymphocytes in the HAM/TSP patients may result from the increased proviral load.

2. Materials and methods

2.1. Subjects

PBL were collected by gradient centrifugation from eight HAM/TSP patients and seven AC. HTLV-I infection was confirmed by Western blot of sera from these cases. The diagnosis of HAM/TSP was made according to neurological symptoms and serological testing for HTLV-I in CSF (Osame et al., 1987). The clinical data of the HAM/TSP patients are summarized in Table 1. Patient 5 had abnormal responses in the lower extremities and extensor plantar responses indicative of corticospinal tract lesion(s) as reported previously (Elovaara et al., 1993; Greten et al., 1998), so that the patient was diagnosed as possible HAM/TSP. Genomic DNA was extracted from PBL by DNA extraction kit (Qiagen, Chatsworth, CA) and used in

Table 1
Clinical profiles of HAM/TSP patients

Patient no.	Age/sex/duration of disease	CSF				MRI		EDSS[b]
		WBC (mm[b])	Protein (mg/dl)	OCB[a]	IgG index	Head	Spine	
1	47/F/3	5	38	+	0.75	Abn[c]	Abn	6.0
2	45/F/5	28	36	+	1.22	Abn	Abn	8.0
3	53/M/13	16	27	+	0.5	NI[d]	NI	6.5
4	46/F/15	1	33	−	1.01	NI	Abn	6.0
5#	71/F	1	40	+	0.5	ND[e]	ND	2.0
6	77/M/9	3	42	+	ND	Abn	NI	6.5
7	47/F/10	12	40	+	0.9	ND	NI	8.0
8	54/M/9	9	30	+	0.63	NI	NI	6.5

[a]Oligoclonal bands; [b]expanded disability severity scale; [c]abnormal; [d]normal; [e]not done.
#The patient is diagnosed as possible HAM/TSP (see Section 2).

a competitive PCR for HTLV-I proviral DNA. The same PBL were analyzed for HTLV-I HTLV-I-specific IFN-γ + CD8 + cells by intracellular cytokine detection.

2.2. Generation of competitor and wild-type pX for quantitative PCR

For a competitor, HTLV-I DNA was amplified with two set of primers; SK43(5′-CGATACCCAGTCTACGT GT-3′; 7359–7378)/SK43C (5′-ACCTGCAGGGATCCG-TCGACACGATGTTAGGCGGGCCGAAC-3′: Non-underlined sequence is at 7446–7417 of HTLV-I) and SK44 (5′-GCCGATAACGCGTCCATCG-3′: 7515–7497)/SK44C (5′-GTCGACGGATCCCTGCAGGTCACGCCC TACTGGCCACCTG-3′: Non-underlined sequence is at 7447–7466) in each reaction (Seiki et al., 1983; Kwok et al., 1988). SK43C and SK44C primers contain 20 base sequences non-homologous to HTLV-I at their 5′ ends

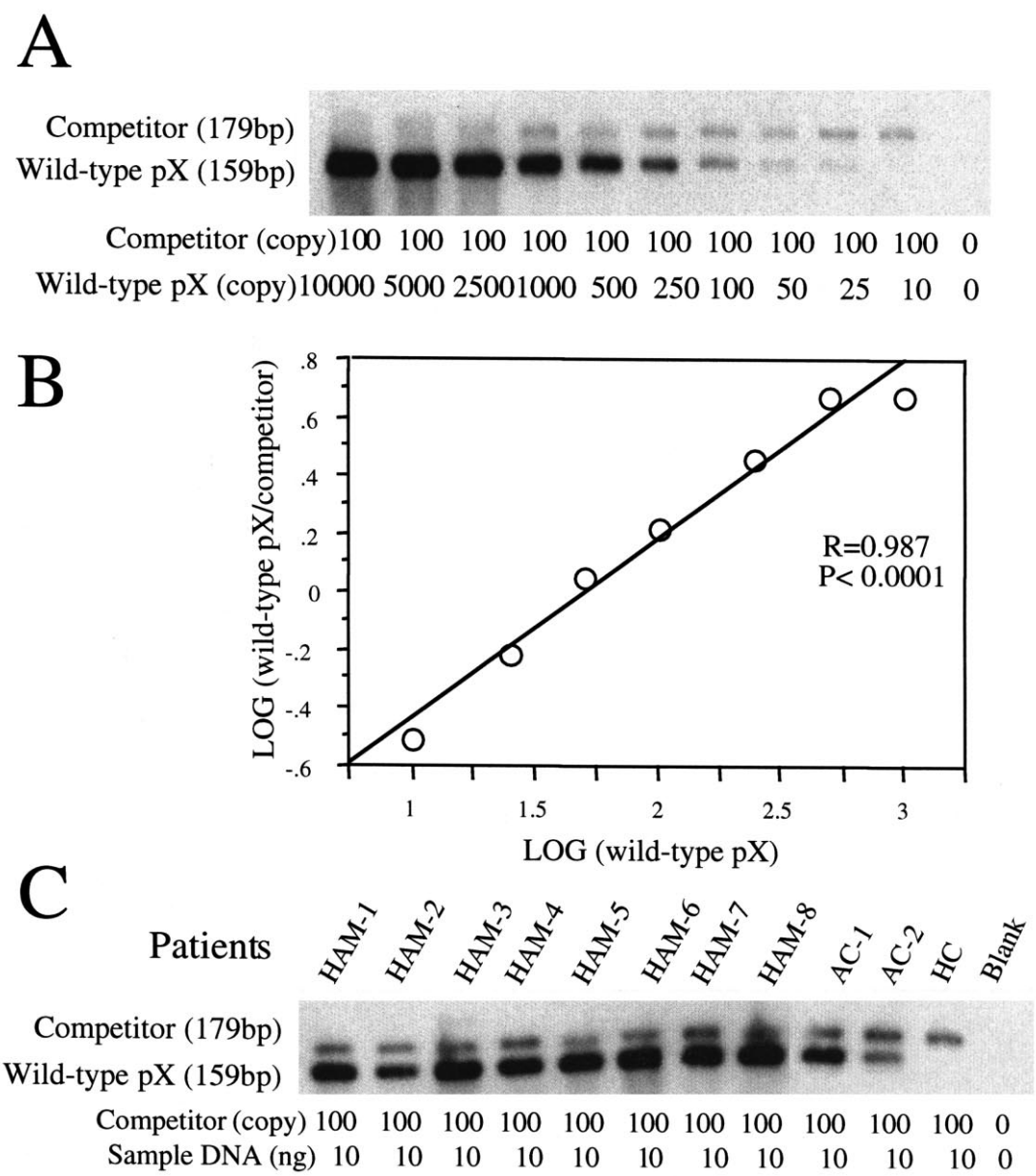

Fig. 1. Quantification of HTLV-I proviral load by the competitive PCR. (A) To generate a standard curve, 100 molecules of competitors and titrated wild-type HTLV-I pX were amplified at 30 cycles in the same tube with SK43/44 primers. (B) The intensity of the band was measured. A ratio of band intensity of wild-type pX to that of competitor was plotted against the intensity of the wild-type pX in logarithmic scale. Linearity was obtained between 1 and 3 of LOG (wild-type pX). (C) Quantification of HTLV-I proviral loads of HAM/TSP patients and asymptomatic HTLV-I carriers (AC). In the samples, 100 copies of competitors and 10 ng of sample DNA were co-amplified with SK43/44 primers. After measuring intensities of the band, the ratio of band intensity of sample's wild-type pX to that of competitor was calculated. Finally, the proviral loads per μg DNA of samples were estimated according to a standard curve as shown in (B). HC, healthy control; blank, PCR was carried out without DNA.

(which is underlined), and these 20 base sequences were complementary to each other. After gel electrophoresis, the products were purified and mixed. The complementary 20 base portion was hybridized. Hybridized DNA was amplified with SK43/44 primers, and the amplified products with the expected size were purified. Wild-type pX from HTLV-I was obtained by amplification with the SK43/44 primers from HTLV-I DNA and purified. The product size of the competitor and the wild-type pX were 179 bp and 159 bp, respectively. After measuring the optical density of both products, the copy number was calculated according to the molecular weight. The products were stored at 1×10^5 molecules/μl in TE buffer (pH = 8.0) at −70°C until use. The concentrated competitor and wild-type pX were serially diluted with distilled water in each experiment.

2.3. Competitive PCR

We put 100 copies of competitor and 10 ng of sample DNA in a tube containing 200 μM dNTP mix, 1.25 U Taq polymerase, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl, 25 pmol SK44, and 25 pmol 5′-digoxigenin-labeled SK43 primer in final volume of 50 μl. For each experiment, we set up other PCR tubes containing 100 copies of competitor and titrated number of wild-type pX to generate a standard curve. PCR amplification was carried out for 30 cycles including denaturation; 94°C/1 min, annealing; 54°C/1 min, extension; 72°C/1 min. Five microliter of the PCR products were run on a 4% NuSieve agarose gel (FMC BioProducts, Rockland, ME). After transfer to a nylon membrane, incorporated digoxigenin in the products were bound with an anti-digoxigenin antibody and signals were detected on an X-ray film by DIG detection system (Boehringer Mannheim, Indianapolis, IN). After exposure, the signals on the film were scanned into a computer and the intensity of the band was analyzed by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). To generate a standard curve, a ratio of the signal intensity of wild-type pX to that of competitor was plotted against the signal intensity of the wild-type pX using a logarithmic scale. A ratio of signal intensity of the sample DNA to that of the competitor was estimated. Finally, the proviral load in the samples per 1 μg of DNA was calculated according to the standard curve.

2.4. Intracellular cytokine detection

Our method for intracellular cytokine detection has been previously described (Kubota et al., 1998). Briefly, 1×10^6 PBL were preincubated in 96-well round-bottom plates in 200 μl of RPMI-1640 medium containing 5% heat inactivated human serum, 2.8 mM of L-glutamine, 14 mM of Hepes buffer, 40 U/ml of penicillin and 40 μg/ml of streptomycin for 4 h, then brefeldin A (Sigma, St. Louis, MO) was added to the wells at a final concentration of 10 μg/ml. After incubation for 10 h at 37°C, cells were harvested and washed in cold staining buffer containing 1% fetal calf serum (FCS) and 0.1% sodium azide in phosphate-buffered saline (PBS) (pH 7.4). Cells were pretreated with 10 μg/μl of human γ-globulin at 4°C for 5 min followed by staining with 0.5 μg of anti-human CD8-TC conjugated antibody (Caltag Laboratories, South San Francisco, CA) at 4°C for 30 min. Cells were washed and fixed with 100 μl of 4% paraformaldehyde in PBS (pH 7.4) at 4°C for 20 min. After fixation, cells were washed and resuspended in 50 μl of permeabilization buffer which contained 0.1% saponin, 1% FCS and 0.1% sodium azide in PBS (pH 7.4). The cells were then pretreated with 2 μg/μl of human γ-globulin at 4°C for 5 min followed by staining with anti-IFN-γ-FITC conjugated antibody (PharMingen, San Diego, CA) at 4°C for 30 min. Cells were washed with permeabilization buffer and finally resuspended in 200 μl of staining buffer. FACScan and CELLQuest computer software were utilized for fluorescent signal detection and the data analysis (Becton-Dickinson, Mountain View, CA). Fifty thousand events were recorded. The percentage of IFN-γ + CD8 + cells in PBL was measured.

3. Results

3.1. Validation of quantitative PCR

To generate a standard curve, we amplified a constant number of competitor and a known number of wild-type HTLV-I pX with the same primers in each tube. We made

Table 2
HAM/TSP patients have higher HTLV-I proviral loads and numbers of IFN-γ + CD8 + cells in PBL than AC

Patient	No.	HTLV-I proviral load (copy/μg DNA)	IFN-γ + CD8 + cells (% in total PBL)
HAM/TSP	1	39,828	1.68
	2	12,109	0.61
	3	33,601	1.25
	4	23,849	0.68
	5#	65,095	1.30
	6	56,137	4.85
	7	15,724	0.89
	8	30,512	0.47
	mean ± SE	34,482 ± 6621*	1.47 ± 0.50**
AC	1	34,217	0.09
	2	2256	0.05
	3	61	0.14
	4	17,755	0.07
	5	1520	0.02
	6	12,408	0.19
	7	273	0.02
	mean ± SE	9784 ± 4824	0.08 ± 0.02

* $P = 0.021$, ** $P = 0.001$, when compared with those in AC by Mann–Whitney U-test. #: The patient has possible HAM/TSP (see Section 2).

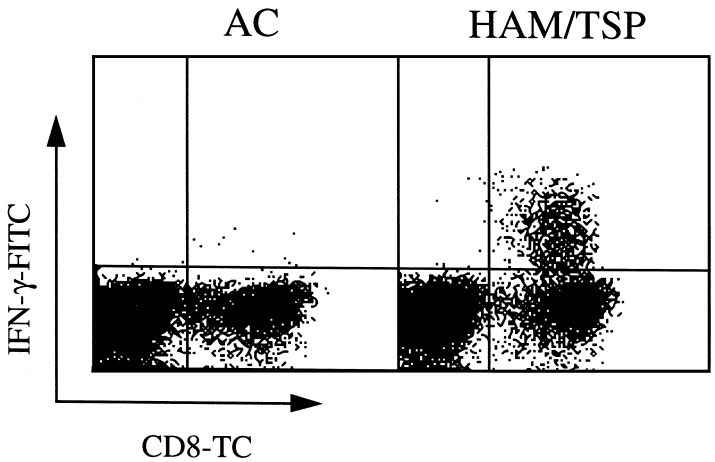

Fig. 2. IFN-γ + CD8 + cells were detected by flow cytometry combined with intracellular cytokine staining. One million PBL were cultured for 4 h without mitogen or IL-2 and followed by a 10-h culture with 10 μg/ml of brefeldin A. Cells were stained with anti-CD8-TC conjugated antibody, then fixed and stained with anti-IFN-γ-FITC conjugated antibody. The IFN-γ + CD8 + lymphocytes in total PBL of the AC and HAM/TSP patient were 0.09 and 4.85%, respectively.

a standard curve in each experiment. Fig. 1A represents a PCR done for a standard curve. A plateau effect of PCR was observed over 3 of LOG (wild-type pX), when plotted LOG (wild-type pX/competitor) to LOG (wild-type pX) (data not shown). When we plotted them between 1 and 3 of LOG (wild-type pX) (Fig. 1B), we could achieve linearity. Therefore, we made a standard curve between 10 and 1000 copies per reaction, and value for R square of the regression curve was over 0.97 in each experiment ($P < 0.005$).

3.2. Quantification of HTLV-I proviral load in HAM / TSP and AC

Fig. 1C represents the quantitative PCR. After measuring the intensity of each band, a ratio of intensity of the sample's wild-type pX to that of the competitor was calculated. The proviral load was estimated according to a standard curve. The average proviral loads were 34,482 and 9784 copy/μg DNA in HAM/TSP and AC group, respectively (Table 2) which was significantly different by the Mann–Whitney U-test ($P = 0.021$).

3.3. Intracellular IFN-γ detection by flow cytometry

Fig. 2 shows a representative flow cytometric analysis for intracellular IFN-γ detection. The HAM/TSP patient had increased IFN-γ + CD8 + cells in his PBL (4.85%), while IFN-γ + CD8 + cells in the AC was 0.09%. In addition, we measured the percentage of IFN-γ + CD8 + cells in PBL of HAM/TSP patient #3 at three time points over 5 years (Table 2). The high proportion of IFN-γ + CD8 + cells was persistent in the patient although the disease was exacerbated during this period. When we compared the percentage of IFN-γ positive cells between eight HAM/TSP patients and seven AC, the average percentage of IFN-γ + CD8 + cells in total PBL was 1.47 and 0.08%, respectively (Table 2). The percentage in HAM/TSP patients was significantly higher than that in AC ($P = 0.001$, by the Mann–Whitney U-test).

3.4. Correlation of the IFN-γ + CD8 + cells with the proviral load

When the percentage of IFN-γ + CD8 + cells in PBL was plotted against the proviral load in all the infected

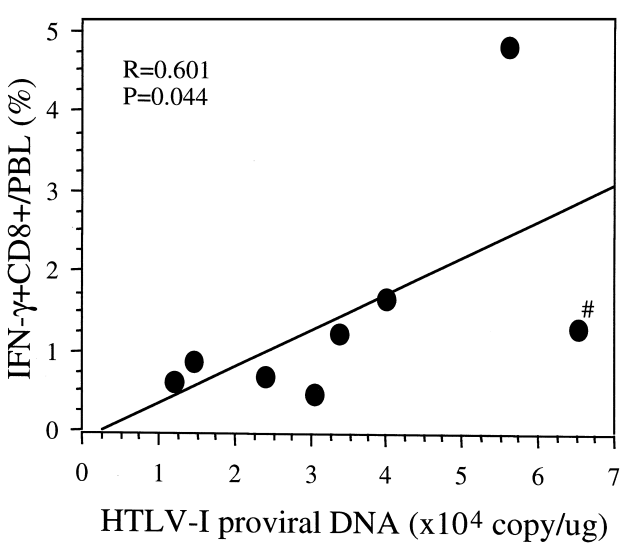

Fig. 3. In the same series samples of eight HAM/TSP patients, HTLV-I proviral load and IFN-γ + CD8 + cells were measured and plotted. X-axis and Y-axis show the proviral load per μg DNA and the percentage of the IFN-γ + CD8 + cells in total PBL, respectively. Positive correlation was observed by Spearman's rank correlation. #: The patient has a possible HAM/TSP (see Section 2).

individuals, a positive correlation was observed in the HAM/TSP patients ($P = 0.044$) by Spearman's rank correlation (Fig. 3). No positive correlation was observed between HTLV-I proviral load and IFN-γ + CD8 + cells in the AC ($P = 0.508$).

4. Discussion

In this study, we demonstrated that HAM/TSP patients have both high proviral loads and increased IFN-γ + CD8 + lymphocytes specific for HTLV-I in the same samples, when compared to AC. The proportion of HTLV-I-specific IFN-γ + CD8 + lymphocytes positively correlate with the proviral load in PBL of the HAM/TSP patients but not in the AC. Moreover, in patient 3, high number of IFN-γ + CD8 + lymphocytes was persistent over 5 years. These data suggest that high levels of both HTLV-I-specific lymphocytes and proviral load are viroimmunological hallmarks in HAM/TSP patients. This high proportion of HTLV-I-specific CD8 + lymphocytes in PBL produced proinflammatory cytokines, such as IFN-γ, through recognition of HTLV-I antigens. We have previously reported that HAM/TSP patients have also increased the number of tumor necrosis factor (TNF)-α + CD8 + lymphocytes in the PBL (Kubota et al., 1998). In the affected lesions of HAM/TSP patients, HTLV-I genome and the gene products as well as MHC class I molecules have been shown (Kubota et al., 1994; Lehky et al., 1995; Moritoyo et al., 1996). Moreover, HTLV-I-specific CTL was also demonstrated in the biopsied spinal cord from a HAM/TSP patient (Levin et al., 1997). Therefore, in the central nervous system, HTLV-I-specific CD8 + lymphocytes may produce proinflammatory cytokines through recognition of HTLV-I antigens, which may result in neuronal damage in patients with HAM/TSP.

In HTLV-I infection, it is well known that peripheral lymphocytes spontaneously proliferate in vitro without mitogen and or exogenous IL-2, and that HAM/TSP patients have higher levels of spontaneous lymphocytes proliferation than do AC (Itoyama et al., 1988; Jacobson et al., 1988; Usuku et al., 1988). This phenomenon has been indicated to consist of a mixed proliferation of both HTLV-I-infected CD4 + cells and HTLV-I-specific CD8 + cells (Eiraku et al., 1992; Machigashira et al., 1997). Therefore, the intensity of the in vitro phenomenon observed in HAM/TSP patients is consistent with our present observation that HAM/TSP patients have high proviral loads and increased HTLV-I-specific CD8 + lymphocytes. The high proviral load in HAM/TSP patients may result from potential sources of viral replication, (1) proliferation of infected cells and (2) new infection to non-infected cells by free virus like as is seen for HIV-1. Southern blotting and linker mediated or inverse PCR have shown oligoclonal proliferation of HTLV-I-infected cells in PBL of infected individuals without malignancy (Furukawa et al., 1992; Wattel et al., 1995). Therefore, it seems likely that HTLV-I preferentially replicates through proliferation of infected cells rather than through new infection by free virus.

In the present study, we have shown that HTLV-I-specific CD8 + lymphocytes positively correlate with the proviral load in PBL of the HAM/TSP patients but not in the AC. This suggests that a high number of HTLV-I-specific lymphocytes in HAM/TSP patients may result from increased proviral loads. In contrast, in HIV-1 infection, a significant inverse relationship was demonstrated between the frequency of HIV-1-specific lymphocytes and the viral load in plasma (Greenough et al., 1997; Ogg et al., 1998). It would be unusual that high viral burden could be persistent despite high CTL response and a high titer of anti-HTLV-I antibody, as are observed in HAM/TSP patients (Osame et al., 1987; Jacobson et al., 1990). These findings suggest that high cellular and humoral immune responses to HTLV-I cannot eliminate the infected cells completely in HAM/TSP patients. It has been shown that HAM/TSP patients have low or trace levels of viral antigens expression in the PBL, when detected by using conventional techniques such as Northern blotting and immunofluorescence, although expression of the viral antigens is readily detected after a short culture of the PBL (Gessain et al., 1990). A possible reason for the high proviral loads despite the high immune responses in PBL of HAM/TSP patients is that some HTLV-I-infected cells expressing viral antigens would be immediately eliminated by the CTL and the remaining infected cells, which are not expressing viral antigens, may survive, so that we could not detect antigens-expressing cells in the circulation. However, Nagasato et al. demonstrated high anti-HTLV-I IgM antibodies in HAM/TSP patients (Nagasato et al., 1991). This evidence, together with the existence of chronically activated CTL in HAM/TSP patients, suggests that immunocompetent cells may be continuously stimulated by infected cells that express viral antigens somewhere in vivo. What mechanisms are involved in keeping that many of the infected cells tend not to express the viral antigens in the circulation, and where and how the virus can replicate in the bodies of HTLV-I-infected individuals must be elucidated (Saiga et al., 1997; Ijichi et al., 1998).

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